

DNA CHIP BASED GENETIC TYPING

Technical Field

This invention relates generally to the field of nucleic acid analysis. In particular, the invention provides a method for typing a target gene, using, *inter alia*, a chip comprising a support suitable for use in nucleic acid hybridization having immobilized thereon an oligonucleotide probe complementary to said target nucleotide sequence and at least one of the following oligonucleotide control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe. Oligonucleotide probes or probe arrays for typing a human leukocyte antigen (HLA) target gene are also provided.

Background Art

Human leukocyte antigens (HLA) are encoded by HLA gene complex located on the short arm of human chromosome six. The human HLA genes are part of the major histocompatibility complex (MHC), a cluster of genes associated with tissue antigens and immune responses. Successful organ transplantation between individuals depends on the degree of acceptance, *i.e.*, histocompatibility, between donor and recipient pairs. Antigens that cause rejection to the transplanted organ are transplantation antigens or histocompatibility antigens. There are more than twenty antigen systems related to rejection reaction in a human body. Among them, the one that can cause strong and acute rejection reaction is called major histocompatibility antigen. Its gene is a cluster of tightly connected genes, called major histocompatibility complex (MHC). It has now being proved that the immune response gene (IR gene) that controls immune response and regulating function is located in MHC. Thus, MHC not only relates to transplantation rejection but also involves widely in induction and regulation of immune response and regulation. HLA genes are located in a region of about 4000 kb located on human chromosome six, occurring about 1/3,000 of the the entire human genome. There are 224 identified HLA loci. The HLA proteins are classified, based on their structures, expression pattern, tissue distribution, and function, into three classes: HLA-I, HLA-II, and HLA-III. Within each gene locus, there are hundreds of alleles.

The proteins encoded by HLA genes play an important role in graft rejection during tissue transplantation. Successful tissue transplantation depends on achieving a degree of

HLA matching between donor and recipient. Thus, HLA typing is necessary for selection of an optimally matched donor. Currently, HLA typing is routinely done in connection with many medical procedures, *e.g.*, organ transplantation, especially bone marrow transplantation. Based on extensive polymorphism in HLA genes of the human population, the role of the proteins encoded by HLA genes in regulating immune response, and codominant expression by both the paternal and maternal genes, HLA typing is also used in predicting susceptibility to diseases, forensic identification, paternity determination, and genetic studies. Accordingly, there is a need for accurate HLA typing methods.

10 Different methods have been used for HLA typing. Currently, HLA genes are typed using serological methods, mixed lymphocyte culture methods (MLC), and DNA sequence-based typing methods.

Serological methods are based on reactions of sera with the HLA proteins on the surface of lymphocytes. Methods based on the principle of serological typing, such as ID-IEF and monoclonal antibody typing method, have been developed to improve specificity and shorten the testing time. Major drawbacks to serological HLA typing are the complexity of the sera, the lack of widespread availability of standard sera necessary to conduct the tests, and that only the already known HLA types, but not new polymorphisms, are detected.

20 In mixed lymphocyte culture (MLC) tests, lymphocytes from one individual (the "responder") are cultured with "stimulating" lymphocytes from another individual. When the stimulating cells are from unrelated persons or family members whose MHC is different from that of the responder, the untreated lymphocytes proliferate; this proliferation is an indicator for non-matching antigens from the individuals. MLC methods are not widely used for the lack of availability of typing cells and complexity of testing procedures.

DNA sequence-based HLA typing methods have been developed to overcome drawbacks with serological or mixed lymphocyte culture methods. One such method involves the use of DNA restriction fragment length polymorphism (RFLP) as a basis for HLA typing. *See* U.S. Pat. No. 4,582,788. Polymorphism in the length of restriction endonuclease digests generated by polymorphism in the HLA genes of the human population in combination with polymerase chain reaction (PCR) technology are used for HLA typing. However, RFLP method fails to differentiate between certain alleles that are

known to exist in the population (e.g., subtypes of HLA-DR4), and thus, cannot be used to distinguish certain combinations of alleles. Moreover, its practical usefulness is limited because the procedures involved take about two weeks to complete and require use of radioactivity.

5 More recently, researchers have established sequence-specific oligonucleotide (SSO) probe hybridization method to perform HLA-II typing. The method entails amplifying a polymorphic region of a HLA locus using PCR, hybridizing the amplified DNA to a sequence-specific oligonucleotide probe(s), and detecting hybrids formed between the amplified DNA and the sequence-specific oligonucleotide probes. This method can
10 identify one or two nucleotide difference between HLA alleles. The drawbacks of this method is the complexity and difficulty of making multiple equivalent membranes for hybridization or reuse of the same membrane after hybridization which currently is not automated due to the high number of alleles under investigation. Although reverse line strip typing method has been developed to improve the SSO method using an enzymatic
15 method for generating signals for detection, the operation of this method is complicated and difficult to get desired results.

Sequence specific primer amplification (PCR-SSP) method for HLA typing utilises the specific sequence sites in PCR primer for PCR amplification of HLA type and analyzes amplified product by electrophoresis. The time required for the test using this method is
20 only 2 to 3 hours. Mytilineos et al., *Hum. Immunol.*, 59: 512-7 (1998). However, for an unknown sample, the method requires a lot of reasearch for testing each specific primer. In addition, it is difficult to obtain high resolution typing for HLA subtypes.

Other DNA sequence-based HLA typing method includes PCR single strand conformation polymorphism (PCR-SSCP) and PCR fingerprinting. DNA
25 sequence-based HLA typing method has made HLA typing more precise and also help identify more HLA alleles.

DNA chip techonology has been widely used for analysing a large number of different DNA sequences or fragments simultaneously on a single DNA chip. The technique allows high-throughput, simulataneous and fast analysis of DNA fragments and
30 requires very minute amount of the target DNA fragment. Because of the complexity of HLA genes, DNA chip can be an ideal tool for use in HLA typing. A few kits and methods have been described. See Kahiwase, *Rinsho Byori Suppl.* 110: 99-106 (1999);

Cao et al., *Rev. Immunogenet.* 1: 177-208 (1999); and Guo et al., *Rev. Immunogenet.* 1: 220-30 (1999).

Disclosure of the Invention

5 In one aspect, the present invention is directed to a method for typing a target gene, which method comprises: a) isolating a target cell comprising a target gene from a suitable sample and obtaining a preparation comprising a target nucleotide sequence that is at least a part of said target gene from said isolated target cell and, optionally another nucleotide sequence not related to said target gene; b) providing a chip comprising a support suitable
10 for use in nucleic acid hybridization having immobilized thereon an oligonucleotide probe complementary to said target nucleotide sequence and at least one of the following oligonucleotide control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe; and c) hybridizing said preparation obtained in step a) to said chip provided in step b) and assessing hybridization
15 between said target nucleotide sequence and/or said another nucleotide sequence and said control probes comprised on said chip to determine the type of said target gene.

 In another aspect, the present invention is directed to an oligonucleotide probe for typing a HLA target gene comprising a nucleotide sequence that: a) hybridizes, under high stringency, with a target HLA nucleotide sequence, or a complementary strand thereof,
20 that is set forth in Table 1; or b) has at least 90% identity to a target HLA nucleotide sequence comprising a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1.

 In still another aspect, the present invention is directed to an array of oligonucleotide probes immobilized on a support for typing a HLA target gene, which array comprises a
25 support suitable for use in nucleic acid hybridization having immobilized thereon a plurality of oligonucleotide probes, at least one of said probes comprising a nucleotide sequence that: a) hybridizes, under high stringency, with a target HLA nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1; or b) has at least 90% identity to a target HLA nucleotide sequence comprising a nucleotide sequence, or a
30 complementary strand thereof, that is set forth in Table 1.

Brief Description of the Drawings

Figure 1 illustrates the results that the leukocyte was captured by the magnetic microbead.

Figure 2 illustrates PCR results from leukocytes isolated using three different types of magnetic microbead.

5 Figure 3 illustrates PCR results from leukocytes isolated using same magnetic microbead.

Figure 4 illustrates hybridization signals on a chip comprising 144 probes.

Figure 5 illustrates DHPLC analysis of two probes: PBH_0303019 and PBH_0301119.

10 Figure 6 illustrates DHPLC analysis of four types of probes: 6a. a very pure probe; 6b. a probe with little impurities; 6c. a probe with high percent impurities; 6d. a very poor probe with very high percent impurities.

Modes of Carrying Out the Invention

15 For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

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As used herein, "a" or "an" means "at least one" or "one or more."

As used herein, "primer" refers to an oligonucleotide that hybridizes to a target sequence, typically to prime the nucleic acid in the amplification process.

As used herein, "probe" refers to an oligonucleotide that hybridizes to a target sequence, typically to facilitate its detection. The term "target sequence" refers to a nucleic acid sequence to which the probe specifically binds. Unlike a primer that is used to prime the target nucleic acid in the amplification process, a probe need not be extended to amplify target sequence using a polymerase enzyme. However, it will be apparent to

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those skilled in the art that probes and primers are structurally similar or identical in many cases.

As used herein, "positive control probe" refers to a probe that hybridizes to conserved or consensus sequences of a group (or family) of target sequences. As used herein, "negative control probe" refers to a probe that comprises a single or multiple basepair change(s) when compared to the positive control probe. Preferably, a negative control probe comprises a single basepair change when compared to the positive control probe. Another exemplary negative control probe is a homologous sequence from an origin that is different from an origin from which the target sequence is derived. In one specific example, two positive control probes, *i.e.*, a stronger one and a weaker one, and a single negative control probe can be used together. The stronger positive control probe and the negative control probe are used to assess overall hybridization efficiency. The weaker positive control probe is used to assess hybridization signals of the testing probes, *i.e.*, the non-control probes whose hybridization is to be assessed. For example, a ratio between hybridization signals of the testing probes and hybridization signals of the weaker positive control probe can be used to derive a range to assess the strength of hybridization of the testing probes.

As used herein, "hybridization control probe" refers to probe(s) that is used to assess overall hybridization efficacy independent of the hybridization between the testing probe and the target sequence. For example, if the target sequence is a HLA sequence, a hybridization control probe can be a sequence unrelated to any HLA sequence, preferably from an origin different from which the target HLA target sequence is derived. The hybridization control probe can be modified with a NH_2 group and be applied (or immobilized) to a chip surface in a same or similar concentration and/or procedure through which other probes, including the testing probes, are applied (or immobilized) to the chip surface. Another labeled probe, *e.g.*, Hexachloro fluorescein (HEX), labeled probe, that is complementary to the hybridization control probe can be added in the overall hybridization solution in a concentration or ratio that is compatible to the concentration or ratio of other probes. Other fluoresceins also can be used here. In this way, the overall hybridization process can be monitored. The hybridization control probe can also be used in guiding or determining locations of probes on the chip surface.

As used herein, "immobilization control probe" refers to probe(s) that is used to assess immobilization process. An immobilization control probe does not participate in

any hybridization reactions. In one example, one end of the immobilization control probe is modified, *e.g.*, with a NH_2 group, to facilitate its immobilization on a chip surface, and the other end of the immobilization control probe is labeled with a detectable label, *e.g.*, HEX.

5 As used herein, "HEX" means Hexachloro fluorescein, one of the Fluoresceins.

As used herein, "complementary" means that two nucleic acid sequences have at least 50% sequence identity. Preferably, the two nucleic acid sequences have at least 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity. "Complementary" also means that two nucleic acid sequences can hybridize under low, middle and/or high stringency condition(s).

As used herein, "substantially complementary" means that two nucleic acid sequences have at least 90% sequence identity. Preferably, the two nucleic acid sequences have at least 95%, 96%, 97%, 98%, 99% or 100% sequence identity. Alternatively, "substantially complementary" means that two nucleic acid sequences can hybridize under high stringency condition(s).

As used herein, "two perfectly matched nucleotide sequences" refers to a nucleic acid duplex wherein the two nucleotide strands match according to the Watson-Crick basepair principle, *i.e.*, A-T and C-G pairs in DNA:DNA duplex and A-U and C-G pairs in DNA:RNA or RNA:RNA duplex, and there is no deletions or additions in either of the sequences in the duplex.

As used herein: "stringency of hybridization" in determining percentage mismatch is as follows:

- 1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C;
- 2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C (also referred to as moderate stringency); and
- 3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures (See generally, Ausubel (Ed.) *Current Protocols in Molecular Biology*, 2.9A. Southern Blotting, 2.9B. Dot and Slot Blotting of DNA and 2.10. Hybridization Analysis of DNA Blots, John Wiley & Sons, Inc. (2000)).

As used herein, "melting temperature" (" T_m ") refers to the midpoint of the temperature range over which nucleic acid duplex, *i.e.*, DNA:DNA, DNA:RNA and

RNA:RNA, is denatured. The T_m of the probe herein means the T_m of the hybridized probe.

As used herein, "assessing" refers to quantitative and/or qualitative determination of the hybrid formed between the probe and the target nucleotide sequence, *e.g.*, obtaining an absolute value for the amount or concentration of the hybrid, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of hybridization. Assessment may be direct or indirect, and the chemical species actually detected need not be the hybrid itself but may, for example, be a derivative thereof, reduction or disappearance of the probe and/or the target nucleotide sequence, or some further substance.

As used herein, "magnetic substance" refers to any substance that has the properties of a magnet, pertaining to a magnet or to magnetism, producing, caused by, or operating by means of, magnetism.

As used herein, "magnetizable substance" refers to any substance that has the property of being interacted with the field of a magnet, and hence, when suspended or placed freely in a magnetic field, of inducing magnetization and producing a magnetic moment. Examples of magnetizable substances include, but are not limited to, paramagnetic, ferromagnetic and ferrimagnetic substances.

As used herein, "paramagnetic substance" refers to the substances where the individual atoms, ions or molecules possess a permanent magnetic dipole moment. In the absence of an external magnetic field, the atomic dipoles point in random directions and there is no resultant magnetization of the substances as a whole in any direction. This random orientation is the result of thermal agitation within the substance. When an external magnetic field is applied, the atomic dipoles tend to orient themselves parallel to the field, since this is the state of lower energy than antiparallel position. This gives a net magnetization parallel to the field and a positive contribution to the susceptibility. Further details on "paramagnetic substance" or "paramagnetism" can be found in various literatures, *e.g.*, at Page 169 – page 171, Chapter 6, in "Electricity and Magnetism" by B.I Bleaney and B. Bleaney, Oxford, 1975.

As used herein, "ferromagnetic substance" refers to the substances that are distinguished by very large (positive) values of susceptibility, and are dependent on the applied magnetic field strength. In addition, ferromagnetic substances may possess a magnetic moment even in the absence of the applied magnetic field, and the retention of

magnetization in zero field is known as "remanence". Further details on "ferromagnetic substance" or "ferromagnetism" can be found in various literatures, *e.g.*, at Page 171 – page 174, Chapter 6, in "Electricity and Magnetism" by B.I Bleaney and B. Bleaney, Oxford, 1975.

5 As used herein, "ferrimagnetic substance" refers to the substances that show spontaneous magnetization, remanence, and other properties similar to ordinary ferromagnetic materials, but the spontaneous moment does not correspond to the value expected for full parallel alignment of the (magnetic) dipoles in the substance. Further details on "ferrimagnetic substance" or "ferrimagnetism" can be found in various
10 literatures, *e.g.*, at Page 519- 524, Chapter 16, in "Electricity and Magnetism" by B.I Bleaney and B. Bleaney, Oxford, 1975.

As used herein, "metal oxide particle" refers to any oxide of a metal in a particle form. Certain metal oxide particles have paramagnetic or super-paramagnetic properties. "Paramagnetic particle" is defined as a particle which is susceptible to the application of
15 external magnetic fields, yet is unable to maintain a permanent magnetic domain. In other words, "paramagnetic particle" may also be defined as a particle that is made from or made of "paramagnetic substances". Non-limiting examples of paramagnetic particles include certain metal oxide particles, *e.g.*, Fe₃O₄ particles, metal alloy particles, *e.g.*, CoTaZr particles.

20 As used herein, "the sample, *e.g.*, the whole blood, is fresh" means that the sample has been obtained or isolated from its natural source within about 12 hours. Preferably, the sample has been obtained or isolated from its natural source within about 10, 5, 4, 3, 2 hours, 1 hour, 30, 20, 10, 5, 2 minutes or 1 minute.

As used herein, "the sample, *e.g.*, the whole blood, is low-temperature conserved"
25 means that the sample has been conserved at a temperature about at or below 0°C.

B. Methods for typing a target gene

In one aspect, the present invention is directed to a method for typing a target gene, which method comprises: a) isolating a target cell comprising a target gene from a suitable
30 sample and obtaining a preparation comprising a target nucleotide sequence that is at least a part of said target gene from said isolated target cell and, optionally another nucleotide sequence not related to said target gene; b) providing a chip comprising a support suitable for use in nucleic acid hybridization having immobilized thereon an oligonucleotide probe

complementary to said target nucleotide sequence and at least one of the following oligonucleotide control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe; and c) hybridizing said preparation obtained in step a) to said chip provided in step b) and assessing hybridization
5 between said target nucleotide sequence and/or said another nucleotide sequence and said control probes comprised on said chip to determine the type of said target gene.

The present methods can be used to type a target gene from any target cell, *e.g.*, a leukocyte. Other exemplary target cells include animal cells, plant cells, fungus cells, bacterium cells, recombinant cells and cultured cells.

10 The present methods can be used to type any target gene, *e.g.*, a human leukocyte antigen (HLA).

Any suitable sample, *e.g.*, blood, saliva, hair and a human tissue that comprises a human nucleic acid, can be used in the present methods. In one example, the blood sample is serum, plasma or whole blood. In another example, the blood sample is fresh or
15 low-temperature conserved whole blood.

The target cell can be isolated from a suitable sample using any suitable methods. For example, the target cell can be isolated from the suitable sample using a magnetic microbead. Preferably, the magnetic microbead has a diameter ranging from about 5 μm to about 200 μm .

20 The magnetic microbeads can be prepared by any suitable methods. For example, the methods disclosed in CN 01/109870.8 or WO02/075309 can be used. Any suitable magnetizable substance can be used to prepare the magnetic microbeads useful in the present methods. No-limiting examples of the magnetizable substances include ferrimagnetic substance, ferromagnetic substance, paramagnetic substance or superparamagnetic substances. In a specific embodiment, the magnetic microbeads
25 comprise a paramagnetic substance, *e.g.*, a paramagnetic metal oxide composition. Preferably, the paramagnetic metal oxide composition is a transition metal oxide or an alloy thereof. Any suitable transition metals can be used, such as iron, nickel, copper, cobalt, manganese, tantalum (Ta), zinc and zirconium (Zr). In a preferred embodiment, the metal oxide composition is Fe_3O_4 or Fe_2O_3 . In another example, the magnetizable
30 substance used in the magnetic microbeads comprises a metal composition. Preferably, the metal composition is a transition metal composition or an alloy thereof such as iron,

nickel, copper, cobalt, manganese, tantalum, zirconium and cobalt-tantalum-zirconium (CoTaZr) alloy.

The magnetic microbeads may be prepared from the available primary beads, from raw materials or from metal oxides that are encapsulated by monomers which when crosslinked form rigid, polymeric coatings as disclosed in U.S. Patent No. 5,834,121. As used herein, "rigid" refers to a polymeric coating that is cross linked to the extent that the polymeric coating stabilizes the metal oxide particle within the coating (i.e. the coating essentially does not swell or dissolve) so that the particle remains enclosed therein. As used herein, "microporous" refers to a resinous polymeric matrix that swells or expands in polar organic solvent. As used herein, "load" is used to mean the capacity of the bead for attachment sites useful for functionalization or derivatization.

Suitable substances which may be incorporated as magnetizable materials, for example, include iron oxides such as magnetite, ferrites of manganese, cobalt, and nickel, hematite and various alloys. Magnetite is the preferred metal oxide. Frequently, metal salts are taught to be converted to metal oxides then either coated with a polymer or adsorbed into a bead comprising a thermoplastic polymer resin having reducing groups thereon. When starting with metal oxide particles to obtain a hydrophobic primary bead, it is necessary to provide a rigid coating of a thermoplastic polymer derived from vinyl monomers, preferably a cross-linked polystyrene that is capable of binding or being bound by a microporous matrix. Magnetic particles may be formed by methods known in the art, e.g., procedures shown in Vandenberg et al., *J. of Magnetism and Magnetic Materials*, 15-18:1117-18 (1980); Matijevic, *Acc. Chem. Res.*, 14:22-29 (1981); and U.S. Patent. Nos. 5,091,206; 4,774,265; 4,554,088; and 4,421,660. Examples of primary beads that may be used in this invention are shown in U.S. Patent. Nos. 5,395,688; 5,318,797; 5,283,079; 5,232,7892; 5,091,206; 4,965,007; 4,774,265; 4,654,267; 4,490,436; 4,336,173; and 4,421,660. Or, primary beads may be obtained commercially from available hydrophobic or hydrophilic beads that meet the starting requirements of size, sufficient stability of the polymeric coating to swell in solvents to retain the paramagnetic particle, and ability to adsorb or absorb the vinyl monomer used to form the enmeshing matrix network. Preferably, the primary bead is a hydrophobic, polystyrene encapsulated, paramagnetic bead. Such polystyrene paramagnetic beads are available from Dynal, Inc. (Lake Success, N.Y.), Rhone Poulenc (France), and SINTEF (Trondheim, Norway). The use of toner

particles or of magnetic particles having a first coating of an unstable polymer which are further encapsulated to produce an exterior rigid polymeric coating is also contemplated.

The preparation of the target nucleotide sequence can comprise a nucleic acid amplification step. The target nucleotide sequence can be obtained via nucleic acid
5 amplification directly from the isolated target cell. Alternatively, the target nucleotide sequence can be obtained via nucleic acid amplification using a nucleic acid template isolated from the isolated target cell. Any suitable nucleic acid amplification step can be used, *e.g.*, polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA) and
10 transcription-mediated amplification (TMA). Preferable the TMA is driven by a T7 promoter.

Also preferably, the PCR is asymmetrical PCR. The two primers used in the asymmetrical PCR can have any suitable ratio, *e.g.*, a ratio ranging from about 1:5 to about 1:200. The two primers used in the asymmetrical PCR can have same or different T_m
15 values. For example, the difference between the T_m value of the two primers used in the asymmetrical PCR can range from about 1°C to about 20°C. In another example, three primers are used in the asymmetrical PCR, two of the primers having same or similar T_m value and the difference between the T_m value of the two primers and that of the third primer ranges from about 1°C to about 20°C. The primers can be straight-chain primers
20 or comprise a hairpin structure. A single or multiple annealing temperatures can be used in the PCR. For example, the difference between the annealing temperatures can range from about 1°C to about 20°C.

The target nucleotide sequence obtained in step a) of the present methods can be single-, double- or triple-stranded. Preferably, the target nucleotide sequence obtained in
25 step a) is single-stranded DNA or RNA. The target nucleotide sequence obtained in step a) of the present methods can be a positive or negative strand. Preferably, the single-stranded DNA or RNA is positive or negative strand. A labeled target nucleotide sequence can be obtained in step a). Preferably, the labeled target nucleotide sequence comprises a fluorescent or biotin label. Also preferably, the another nucleotide sequence
30 can be complementary to the positive control probe, the negative control probe or the hybridization control probe comprised on the chip.

The probes comprised on the chip can be positive-stranded or negative-stranded probes. The probes comprised on the chip can be modified. Exemplary probe

modifications include 5'-NH₂ modification, 5'-SH modification, 5'-polyT(or A, C or G) modification, 5'-biotin modification, 3'-NH₂ modification, 3'-SH modification, 3'-polyT(or A, C or G) modification and 3'-biotin modification.

5 The chip used in the present methods can comprise any suitable types or number of probes. For example, the chip can comprise 1-500 different types of probes. In another example, the chip can comprise multiple arrays of probes and each array comprises 1-400 different types of probes.

The probes can be immobilized on the chip at any suitable temperature, *e.g.*, a temperature ranging from about 40°C to about 100°C. The chip can be modified.
10 Exemplary chip modifications include CHO, NH₂, poly-lysine, SH, BSA, streptavidin, agarose gel and polyacrylamide gel modification.

Thesequence, purity or terminal modification of the probes can be assessed. Preferbaly, the sequence, purity or terminal modification of the probes is assessed via DHPLC.

15 Any suitable copies or number of a probe can be immobilized on the chip. For example, multiple copies of a probe, *e.g.*, 1-10 copies of a probe, can be immobilized on the chip.

The multiple copies or number of probes can be immobilized on the chip according to any suitable patterns. For example, the multiple copies or number of probes can be
20 immobilized adjacently or separately on the chip. Preferably, the multiple copies of a positive control probe are immobilized on the chip and the variations in the length and sequence of the immobilized positive control probes, when hybridized with the target nucleotide sequence or the another nucleotide sequence in the preparation provided in step a), create a group of hybridization signals having strong-to-weak or weak-to-strong orderly
25 magnitude.

Any suitable positive control probes can be used in the present methods. Preferably, the positive control probe is complementary to a portion of the target nucleotide sequence, a nucleotide sequence amplified synchronically with the target nucleotide sequence or a synthetic nucleotide sequence.

30 Any suitable negative control probes can be used in the present methods. Preferably, the negative control probe has about 1-3 basepair mismatches when compared to the positive control probe.

Any suitable hybridization control probes can be used in the present methods. Preferably, the hybridization control probe is complementary to a synthetic nucleotide sequence not related to the target gene. More preferably, the hybridization control probe is complementary to a synthetic labeled nucleotide sequence or has about 1-2 basepair mismatches when compared to the synthetic labeled nucleotide sequence.

Any suitable immobilization control probes can be used in the present methods. Preferably, the immobilization control probe does not generate any hybridization signal. Generally, the immobilization control probe is an internal control probe for the quality control of the chemical modified slides, spot process, immobilization procedure, etc. It does not hybridize with the target nucleic acids. In one specific embodiment, one end of the immobilization control probe is chemically modified and the other end of the immobilization control probe has a detectable label.

The chip used in the present methods can comprise any, some or all of the positive control probe, the negative control probe, the hybridization control probe and the immobilization control probe. In one specific embodiment, the chip comprises a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe. The positive control probe, the negative control probe, the hybridization control probe and/or the immobilization control probe can be immobilized on the chip in any suitable pattern. For example, the positive control probe, the negative control probe, the hybridization control probe and the immobilization control probe can be immobilized on the four corners of the chip, in the center of the chip or have any suitable orderly or random immobilization pattern.

The hybridization reaction in step c) can be conducted in any suitable hybridization solution, *e.g.*, a hybridization solution comprising sodium chloride/sodium citrate (SSC) and a surfactant. The hybridization solution can comprise any suitable concentration of SSC, *e.g.*, from about 3X to about 10X SSC. Any suitable surfactant, *e.g.*, sodium dodecyl sulfate (SDS), Triton X100 and sodium lauryl sarcosine (SLS), can be used. The hybridization solution can comprise any suitable concentration of surfactant, *e.g.*, a concentration ranging from about 0.05% (w/w) to about 5% (w/w).

The hybridization reaction in step c) can be conducted at any suitable temperature, *e.g.*, at a temperature ranging from about 42°C to about 70°C.

The present methods can further comprise a washing step after the hybridization reaction. Any suitable washing solution can be used. For example, the washing step can

be conducted in a washing solution comprising a surfactant having a concentration ranging from about 0% (w/w) to about 2% (w/w). The washing step can be conducted for any suitable time, *e.g.*, for a time ranging from about 5 minutes to about 30 minutes.

5 The immobilization efficiency of various probes can be assessed by any suitable methods. For example, the immobilization efficiency can be assessed by analyzing a signal from the immobilization control probe. The immobilization control probe can carry a detectable label, *e.g.*, a fluorescence molecule.

10 The overall hybridization efficiency, including hybridization involving the oligonucleotide probe complementary to the target nucleotide sequence and various control probes, can be assessed by any suitable methods. For example, the overall hybridization efficiency can be assessed by analyzing the hybridization between the hybridization control probe and a labeled synthetic nucleotide sequence not related to the target gene.

15 The overall hybridization specificity, including hybridization involving the oligonucleotide probe complementary to the target nucleotide sequence and various control probes, can be assessed by any suitable methods. For example, the hybridization specificity can be assessed by analyzing the ratio between the hybridization signal involving the positive control probe and the hybridization signal involving the negative control probe, and the ratio between the hybridization signal involving the positive hybridization control probe and the hybridization signal involving the negative hybridization control probe, and increased ratios indicating the increased hybridization specificity.

20 Positive signal(s) can be determined based on any suitable criteria. For example, in hybridizations involving a group of closely related probes, a positive signal(s) can be determined based on the following criteria: a) the ratio of the hybridization signal over background noise is more than 3; b) the ratio of the hybridization signal over a relevant positive control probe hybridization signal is within a predetermined range; c) comparing hybridization signals of all probes giving positive signals based on the steps of a) and b), or hybridization signals of two probes giving two strongest hybridization signals when
30 only one probe giving positive signal based on the steps of a) and b), to determine whether the signal is positive or negative; and d) there are 2 or less than 2 positive signals involving the group of closely related probes.

The group of closely related probes can be based on any suitable criteria. For example, a group of probes designed to assess variation at a particular genetic locus can be used as a group of closely related probes. The variation to be assessed can be single or multiple basepair change(s). Normally, the basepair change(s) are located within the length of a probe, *e.g.*, within 20 bps.

The predetermined range as described in the above b) can be different for different probes. The range can be obtained through empirical studies. For example, the range can be obtained by conducting multiple, *e.g.*, hundreds, hybridization experiments using known, standard targets and/or probes.

The present methods can be used to type any target gene. For example, the present methods can be used to type a HLA gene using an oligonucleotide probe that is complementary to the target HLA gene. Preferably, the oligonucleotide probe comprises a nucleotide sequence that: a) hybridizes, under high stringency, with a target HLA nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1; or b) has at least 90% identity to a target HLA nucleotide sequence comprising a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1. Also preferably, the oligonucleotide probe comprises a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1. The chip can comprise some or all nucleotide sequences, or a complementary strand thereof, that are set forth in Table 1.

C. Oligonucleotide probes and probe arrays for typing a HLA target gene

In another aspect, the present invention is directed to an oligonucleotide probe for typing a HLA target gene comprising a nucleotide sequence that: a) hybridizes, under high stringency, with a target HLA nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1; or b) has at least 90% identity to a target HLA nucleotide sequence comprising a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1. The oligonucleotide probe can comprise DNA, RNA, PNA or a derivative thereof. Preferably, the probe comprises a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1. The probe can be labeled. Exemplary labels include a chemical, an enzymatic, an immunogenic, a radioactive, a fluorescent, a luminescent and a FRET label.

The oligonucleotide probes can be produced by any suitable method. For example, the probes can be chemically synthesized (See generally, Ausubel (Ed.) *Current Protocols*

in *Molecular Biology, 2.11. Synthesis and purification of oligonucleotides*, John Wiley & Sons, Inc. (2000)), isolated from a natural source, produced by recombinant methods or a combination thereof. Synthetic oligonucleotides can also be prepared by using the triester method of Matteucci et al., *J. Am. Chem. Soc.*, **3**:3185-3191 (1981). Alternatively, automated synthesis may be preferred, for example, on a Applied Biosynthesis DNA synthesizer using cyanoethyl phosphoramidite chemistry. Preferably, the probes are chemically synthesized.

Suitable bases for preparing the oligonucleotide probes of the present invention may be selected from naturally occurring nucleotide bases such as adenine, cytosine, guanine, uracil, and thymine. It may also be selected from nonnaturally occurring or "synthetic" nucleotide bases such as 8-oxo-guanine, 6-mercaptoguanine, 4-acetylcytidine, 5-(carboxyhydroxyethyl) uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl-2-thioridine, 5-carboxymethylaminomethyl uridine, dihydrouridine, 2'-O-methylpseudouridine, beta-D-galactosylqueosine, 2'-O-methylguanosine, inosine, N⁶-isopentenyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N⁶-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, beta-D-mannosylqueosine, 5-methoxycarbonylmethyluridine, 5-methoxyuridine, 2-methylthio-N⁶-isopentenyladenosine, N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine, N-((9-beta-D-ribofuranosylpurine-6-yl) N-methylcarbamoyl) threonine, uridine-5-oxyacetic acid methyl ester, uridine-5-oxyacetic acid, wybutosine, pseudouridine, queosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 2-thiouridine, 5-methyluridine, N-((9-beta-D-ribofuranosylpurine-6-yl) carbamoyl) threonine, 2'-O-methyl-5-methyluridine, 2'-O-methyluridine, wybutosine, and 3-(3-amino-3-carboxypropyl) uridine.

Likewise, chemical analogs of oligonucleotides (e.g., oligonucleotides in which the phosphodiester bonds have been modified, e.g., to the methylphosphonate, the phosphotriester, the phosphorothioate, the phosphorodithioate, or the phosphoramidate) may also be employed. Protection from degradation can be achieved by use of a "3'-end cap" strategy by which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3' end of the oligonucleotide (Shaw et al., *Nucleic Acids Res.*, **19**:747 (1991)). Phosphoramidates, phosphorothioates, and methylphosphonate linkages all function adequately in this manner. More extensive modification of the phosphodiester

backbone has been shown to impart stability and may allow for enhanced affinity and increased cellular permeation of oligonucleotides (Milligan et al., J. Med. Chem., 36:1923 (1993)). Many different chemical strategies have been employed to replace the entire phosphodiester backbone with novel linkages. Backbone analogues include
5 phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, boranophosphate, phosphotriester, formacetal, 3'-thioformacetal, 5'-thioformacetal, 5'-thioether, carbonate, 5'-N-carbamate, sulfate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite, sulfoxide, sulfide, hydroxylamine, methylene (methylimino) (MMI) or methyleneoxy (methylimino) (MOMI) linkages. Phosphorothioate and
10 methylphosphonate-modified oligonucleotides are particularly preferred due to their availability through automated oligonucleotide synthesis. The oligonucleotide may be a "peptide nucleic acid" such as described by (Milligan et al., J. Med. Chem., 36:1923 (1993)). The only requirement is that the oligonucleotide probe should possess a sequence at least a portion of which is capable of binding to a portion of the sequence of a
15 target DNA molecule.

Hybridization probes can be of any suitable length. There is no lower or upper limits to the length of the probe, as long as the probe hybridizes to the HLA target nucleic acids and functions effectively as a probe (e.g., facilitates detection). The probes of the present invention can be as short as 50, 40, 30, 20, 15, or 10 nucleotides, or shorter. Likewise, the
20 probes can be as long as 20, 40, 50, 60, 75, 100 or 200 nucleotides, or longer, e.g., to the full length of the HLA target sequence. Generally, the probes will have at least 14 nucleotides, preferably at least 18 nucleotides, and more preferably at least 20 to 30 nucleotides of either of the complementary target nucleic acid strands and does not contain any hairpin secondary structures. In specific embodiments, the probe can have a length of
25 at least 30 nucleotides or at least 50 nucleotides. If there is to be complete complementarity, i.e., if the strand contains a sequence identical to that of the probe, the duplex will be relatively stable under even stringent conditions and the probes may be short, i.e., in the range of about 10-30 base pairs. If some degree of mismatch is expected in the probe, i.e., if it is suspected that the probe would hybridize to a variant region, or to
30 a group of sequences such as all species within a specific genus, the probe may be of greater length (i.e., 15-40 bases) to balance the effect of the mismatch(es).

The probe need not span the entire HLA target gene. Any subset of the target region that has the potential to specifically identify HLA target or allele can be used.

Consequently, the nucleic acid probe may hybridize to as few as 8 nucleotides of the target region. Further, fragments of the probes may be used so long as they are sufficiently characteristic of the HLA target gene to be typed.

5 The probe should be able to hybridize with a HLA target nucleotide sequence that is at least 8 nucleotides in length under low stringency. Preferably, the probe hybridizes with a a HLA target nucleotide sequence under middle or high stringency.

In still another aspect, the present invention is directed to an array of oligonucleotide probes immobilized on a support for typing a HLA target gene, which array comprises a support suitable for use in nucleic acid hybridization having immobilized thereon a
10 plurality of oligonucleotide probes, at least one of said probes comprising a nucleotide sequence that: a) hybridizes, under high stringency, with a target HLA nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1; or b) has at least 90% identity to a target HLA nucleotide sequence comprising a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1.

15 The plurality of probes can comprise DNA, RNA, PNA or a derivative thereof. At least one or some of the probes can comprise a nucleotide sequence, or a complementary strand thereof, that is set forth in Table 1. Preferably, probe arrays comprise all of the nucleotide sequences, or a complementary strand thereof, that are set forth in Table 1. At least one, some or all of the probes can be labeled. Exemplary labels include a chemical,
20 an enzymatic, an immunogenic, a radioactive, a fluorescent, a luminescent and a FRET label. Any suitable support, e.g., a silicon, a plastic, a glass, a ceramic, a rubber, and a polymer surface, can be used.

D. Assay formats

25 Immobilization of Probes

The present methods, probes and probe arrays can be used in solution. Preferably, it is conducted in chip format, e.g., by using the probe(s) immobilized on a solid support.

The probes can be immobilized on any suitable surface, preferably, a solid support, such as silicon, plastic, glass, ceramic, rubber, or polymer surface. The probe may also be
30 immobilized in a 3-dimensional porous gel substrate, e.g., Packard HydroGel chip (Broude et al., *Nucleic Acids Res.*, 29(19):E92 (2001)).

For an array-based assay, the probes are preferably immobilized to a solid support such as a "biochip". The solid support may be biological, nonbiological, organic,

inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc.

A microarray biochip containing a library of probes can be prepared by a number of well known approaches including, for example, light-directed methods, such as VLSIPS™ described in U.S. Patent Nos. 5,143,854, 5,384,261 or 5,561,071; bead based methods such as described in U.S. Patent No. 5,541,061; and pin based methods such as detailed in U.S. Patent No. 5,288,514. U.S. Patent No. 5,556,752, which details the preparation of a library of different double stranded probes as a microarray using the VLSIPS™, is also suitable for preparing a library of hairpin probes in a microarray.

Flow channel methods, such as described in U.S. Patent Nos. 5,677,195 and 5,384,261, can be used to prepare a microarray biochip having a variety of different probes. In this case, certain activated regions of the substrate are mechanically separated from other regions when the probes are delivered through a flow channel to the support. A detailed description of the flow channel method can be found in U.S. Patent No. 5,556,752, including the use of protective coating wetting facilitators to enhance the directed channeling of liquids through designated flow paths.

Spotting methods also can be used to prepare a microarray biochip with a variety of probes immobilized thereon. In this case, reactants are delivered by directly depositing relatively small quantities in selected regions of the support. In some steps, of course, the entire support surface can be sprayed or otherwise coated with a particular solution. In particular formats, a dispenser moves from region to region, depositing only as much probe or other reagent as necessary at each stop. Typical dispensers include micropipettes, nanopipettes, ink-jet type cartridges and pins to deliver the probe containing solution or other fluid to the support and, optionally, a robotic system to control the position of these delivery devices with respect to the support. In other formats, the dispenser includes a series of tubes or multiple well trays, a manifold, and an array of delivery devices so that various reagents can be delivered to the reaction regions simultaneously. Spotting methods are well known in the art and include, for example, those described in U.S. Patent Nos. 5,288,514, 5,312,233 and 6,024,138. In some cases, a combination of flow channels and "spotting" on predefined regions of the support also can be used to prepare microarray biochips with immobilized probes.

A solid support for immobilizing probes is preferably flat, but may take on alternative surface configurations. For example, the solid support may contain raised or depressed

regions on which probe synthesis takes place or where probes are attached. In some embodiments, the solid support can be chosen to provide appropriate light-absorbing characteristics. For example, the support may be a polymerized Langmuir Blodgett film, glass or functionalized glass, Si, Ge, GaAs, GaP, SiO₂, SiN₄, modified silicon, or any one
 5 of a variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof. Other suitable solid support materials will be readily apparent to those of skill in the art.

The surface of the solid support can contain reactive groups, which include carboxyl, amino, hydroxyl, thiol, or the like, suitable for conjugating to a reactive group associated
 10 with an oligonucleotide or a nucleic acid. Preferably, the surface is optically transparent and will have surface Si--OH functionalities, such as those found on silica surfaces.

The probes can be attached to the support by chemical or physical means such as through ionic, covalent or other forces well known in the art. Immobilization of nucleic acids and oligonucleotides can be achieved by any means well known in the art (*see, e.g.,*
 15 Dattagupta et al., *Analytical Biochemistry*, 177:85-89(1989); Saiki et al., *Proc. Natl. Acad. Sci. USA*, 86:6230-6234(1989); and Gravitt et al., *J. Clin. Micro.*, 36:3020-3027(1998)).

The probes can be attached to a support by means of a spacer molecule, *e.g.*, as described in U.S. Patent No. 5,556,752 to Lockhart et al., to provide space between the double stranded portion of the probe as may be helpful in hybridization assays. A spacer
 20 molecule typically comprises between 6-50 atoms in length and includes a surface attaching portion that attaches to the support. Attachment to the support can be accomplished by carbon-carbon bonds using, for example, supports having (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide as the solid support). Siloxane bonding can be formed by
 25 reacting the support with trichlorosilyl or trialkoxysilyl groups of the spacer. Aminoalkylsilanes and hydroxyalkylsilanes, bis(2-hydroxyethyl)-aminopropyltriethoxysilane, 2-hydroxyethylaminopropyltriethoxysilane, aminopropyltriethoxysilane or hydroxypropyltriethoxysilane are useful are surface attaching groups.

30 The spacer can also include an extended portion or longer chain portion that is attached to the surface-attaching portion of the probe. For example, amines, hydroxyl, thiol, and carboxyl groups are suitable for attaching the extended portion of the spacer to the surface-attaching portion. The extended portion of the spacer can be any of a variety

of molecules which are inert to any subsequent conditions for polymer synthesis. These longer chain portions will typically be aryl acetylene, ethylene glycol oligomers containing 2-14 monomer units, diamines, diacids, amino acids, peptides, or combinations thereof.

In some embodiments, the extended portion of the spacer is a polynucleotide or the entire spacer can be a polynucleotide. The extended portion of the spacer also can be constructed of polyethyleneglycols, polynucleotides, alkylene, polyalcohol, polyester, polyamine, polyphosphodiester and combinations thereof. Additionally, for use in synthesis of probes, the spacer can have a protecting group attached to a functional group (e.g., hydroxyl, amino or carboxylic acid) on the distal or terminal end of the spacer (opposite the solid support). After deprotection and coupling, the distal end can be covalently bound to an oligomer or probe.

The present method can be used to analyze a single sample with a single probe at a time. Preferably, the method is conducted in high-throughput format. For example, a plurality of samples can be analyzed with a single probe simultaneously, or a single sample can be analyzed using a plurality of probes simultaneously. More preferably, a plurality of samples can be analyzed using a plurality of probes simultaneously.

Hybridization Conditions

Hybridization can be carried out under any suitable technique known in the art. It will be apparent to those skilled in the art that hybridization conditions can be altered to increase or decrease the degree of hybridization, the level of specificity of the hybridization, and the background level of non-specific binding (i.e., by altering hybridization or wash salt concentrations or temperatures). The hybridization between the probe and the target nucleotide sequence can be carried out under any suitable stringencies, including high, middle or low stringency. Typically, hybridizations will be performed under conditions of high stringency.

Hybridization between the probe and target nucleic acids can be homogenous, e.g., typical conditions used in molecular beacons (Tyagi S. et al., *Nature Biotechnology*, 14:303-308 (1996); and U.S. Patent No. 6,150,097) and in hybridization protection assay (Gen-Probe, Inc) (U. S. Patent No. 6,004,745), or heterogeneous (typical conditions used in different type of nitrocellulose based hybridization and those used in magnetic bead based hybridization).

The target polynucleotide sequence may be detected by hybridization with an oligonucleotide probe that forms a stable hybrid with that of the target sequence under high to low stringency hybridization and wash conditions. An advantage of detection by hybridization is that, depending on the probes used, additional specificity is possible. If it is expected that the probes will be completely complementary (i.e., about 99% or greater) to the target sequence, high stringency conditions will be used. If some mismatching is expected, for example, if variant strains are expected with the result that the probe will not be completely complementary, the stringency of hybridization may be lessened. However, conditions are selected to minimize or eliminate nonspecific hybridization.

Conditions those affect hybridization and those select against nonspecific hybridization are known in the art (Molecular Cloning A Laboratory Manual, second edition, J. Sambrook, E. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, 1989). Generally, lower salt concentration and higher temperature increase the stringency of hybridization. For example, in general, stringent hybridization conditions include incubation in solutions that contain approximately 0.1XSSC, 0.1% SDS, at about 65°C incubation/wash temperature. Middle stringent conditions are incubation in solutions that contain approximately 1-2XSSC, 0.1% SDS and about 50°C - 65°C incubation/wash temperature. The low stringency conditions are 2XSSC and about 30°C - 50°C.

An alternate method of hybridization and washing is first to carry out a low stringency hybridization (5XSSPE, 0.5% SDS) followed by a high stringency wash in the presence of 3M tetramethyl-ammonium chloride (TMAC). The effect of the TMAC is to equalize the relative binding of A-T and G-C base pairs so that the efficiency of hybridization at a given temperature corresponds more closely to the length of the polynucleotide. Using TMAC, it is possible to vary the temperature of the wash to achieve the level of stringency desired (Wood et al., *Proc. Natl. Acad. Sci. USA*, 82:1585-1588 (1985)).

A hybridization solution may contain 25% formamide, 5XSSC, 5XDenhardt's solution, 100 µg/ml of single stranded DNA, 5% dextran sulfate, or other reagents known to be useful for probe hybridization.

30

Detection of the Hybrid

Detection of hybridization between the probe and the target HLA nucleic acids can be carried out by any method known in the art, e.g., labeling the probe, the secondary probe,

the target nucleic acids or some combination thereof, and are suitable for purposes of the present invention. Alternatively, the hybrid may be detected by mass spectroscopy in the absence of detectable label (e.g., U.S. Patent No. 6,300,076).

5 The detectable label is a moiety that can be detected either directly or indirectly after the hybridization. In other words, a detectable label has a measurable physical property (e.g., fluorescence or absorbance) or is participant in an enzyme reaction. Using direct labeling, the target nucleotide sequence or the probe is labeled, and the formation of the hybrid is assessed by detecting the label in the hybrid. Using indirect labeling, a secondary probe is labeled, and the formation of the hybrid is assessed by the detection of
10 a secondary hybrid formed between the secondary probe and the original hybrid.

Methods of labeling probes or nucleic acids are well known in the art. Suitable labels include fluorophores, chromophores, luminophores, radioactive isotopes, electron dense reagents, FRET (fluorescence resonance energy transfer), enzymes and ligands having specific binding partners. Particularly useful labels are enzymatically active
15 groups such as enzymes (Wisdom, *Clin. Chem.*, 22:1243 (1976)); enzyme substrates (British Pat. No. 1,548,741); coenzymes (U.S. Patent Nos. 4,230,797 and 4,238,565) and enzyme inhibitors (U.S. Patent No. 4,134,792); fluorescers (Soini and Hemmila, *Clin. Chem.*, 25:353 (1979)); chromophores including phycobiliproteins, luminescers such as chemiluminescers and bioluminescers (Gorus and Schram, *Clin. Chem.*, 25:512 (1979)
20 and *ibid*, 1531); specifically bindable ligands, i.e., protein binding ligands; antigens; and residues comprising radioisotopes such as ^3H , ^{35}S , ^{32}P , ^{125}I , and ^{14}C . Such labels are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., antibodies, enzymes, substrates, coenzymes and inhibitors). Ligand labels are also useful for solid phase capture of the
25 oligonucleotide probe (i.e., capture probes). Exemplary labels include biotin (detectable by binding to labeled avidin or streptavidin) and enzymes, such as horseradish peroxidase or alkaline phosphatase (detectable by addition of enzyme substrates to produce a colored reaction product).

For example, a radioisotope-labeled probe or target nucleic acid can be detected by
30 autoradiography. Alternatively, the probe or the target nucleic acid labeled with a fluorescent moiety can be detected by fluorimetry, as is known in the art. A hapten or ligand (e.g., biotin) labeled nucleic acid can be detected by adding an antibody or an antibody pigment to the hapten or a protein that binds the labeled ligand (e.g., avidin).

As a further alternative, the probe or nucleic acid may be labeled with a moiety that requires additional reagents to detect the hybridization. If the label is an enzyme, the labeled nucleic acid, e.g., DNA, is ultimately placed in a suitable medium to determine the extent of catalysis. For example, a cofactor-labeled nucleic acid can be detected by
5 adding the enzyme for which the label is a cofactor and a substrate for the enzyme. Thus, if the enzyme is a phosphatase, the medium can contain nitrophenyl phosphate and one can monitor the amount of nitrophenol generated by observing the color. If the enzyme is a beta-galactosidase, the medium can contain o-nitro-phenyl-D-galacto-pyranoside, which also liberates nitrophenol. Exemplary examples of the latter include, but are not limited
10 to, beta-galactosidase, alkaline phosphatase, papain and peroxidase. For *in situ* hybridization studies, the final product of the substrate is preferably water insoluble. Other labels, e.g., dyes, will be evident to one having ordinary skill in the art.

The label can be linked directly to the DNA binding ligand, e.g., acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines, by direct
15 chemical linkage such as involving covalent bonds, or by indirect linkage such as by the incorporation of the label in a microcapsule or liposome, which in turn is linked to the binding ligand. Methods by which the label is linked to a DNA binding ligand such as an intercalator compound are well known in the art and any convenient method can be used. Representative intercalating agents include mono-or bis-azido aminoalkyl methidium or
20 ethidium compounds, ethidium monoazide ethidium diazide, ethidium dimer azide (Mitchell et al., *J. Am. Chem. Soc.*, 104:4265 (1982)), 4-azido-7-chloroquinoline, 2-azidofluorene, 4'-aminomethyl-4,5'-dimethylangelicin, 4'-aminomethyl-trioxsalen (4'-aminomethyl-4,5',8-trimethyl-psoralen), 3-carboxy-5- or -8-amino- or -hydroxy-psoralen. A specific nucleic acid binding azido compound has been described
25 by Forster et al., *Nucleic Acid Res.*, 13:745 (1985). Other useful photoreactable intercalators are the furocoumarins which form (2+2) cycloadducts with pyrimidine residues. Alkylating agents also can be used as the DNA binding ligand, including, for example, bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin and norphillin A. Particularly useful photoreactive
30 forms of intercalating agents are the azido intercalators. Their reactive nitrenes are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products (White et al., *Meth. Enzymol.*, 46:644 (1977)).

The probe may also be modified for use in a specific format such as the addition of 10-100 T residues for reverse dot blot or the conjugation to bovine serum albumin or immobilization onto magnetic beads.

When detecting hybridization by an indirect detection method, a detectably labeled
5 second probe(s) can be added after initial hybridization between the probe and the target or during hybridization of the probe and the target. Optionally, the hybridization conditions may be modified after addition of the secondary probe. After hybridization, unhybridized secondary probe can be separated from the initial probe, for example, by washing if the initial probe is immobilized on a solid support. In the case of a solid support, detection of
10 label bound to locations on the support indicates hybridization of a target nucleotide sequence in the sample to the probe.

The detectably labeled secondary probe can be a specific probe. Alternatively, the detectably labeled probe can be a degenerate probe, *e.g.*, a mixture of sequences such as whole genomic DNA essentially as described in U.S. Patent No. 5,348,855. In the latter
15 case, labeling can be accomplished with intercalating dyes if the secondary probe contains double stranded DNA. Preferred DNA-binding ligands are intercalator compounds such as those described above.

A secondary probe also can be a library of random nucleotide probe sequences. The length of a secondary probe should be decided in view of the length and composition of the
20 primary probe or the target nucleotide sequence on the solid support that is to be detected by the secondary probe. Such a probe library is preferably provided with a 3' or 5' end labeled with photoactivatable reagent and the other end loaded with a detection reagent such as a fluorophore, enzyme, dye, luminophore, or other detectably known moiety.

The particular sequence used in making the labeled nucleic acid can be varied. Thus,
25 for example, an amino-substituted psoralen can first be photochemically coupled with a nucleic acid, the product having pendant amino groups by which it can be coupled to the label, *i.e.*, labeling is carried out by photochemically reacting a DNA binding ligand with the nucleic acid in the test sample. Alternatively, the psoralen can first be coupled to a label such as an enzyme and then to the nucleic acid.

30 Advantageously, the DNA binding ligand is first combined with label chemically and thereafter combined with the nucleic acid probe. For example, since biotin carries a carboxyl group, it can be combined with a furocoumarin by way of amide or ester formation without interfering with the photochemical reactivity of the furocoumarin or the

biological activity of the biotin. Aminomethylangelicin, psoralen and phenanthridium derivatives can similarly be linked to a label, as can phenanthridium halides and derivatives thereof such as aminopropyl methidium chloride (Hertzberg et al, *J. Amer. Chem. Soc.*, 104:313 (1982)). Alternatively, a bifunctional reagent such as dithiobis succinimidyl propionate or 1,4-butanediol diglycidyl ether can be used directly to couple the DNA binding ligand to the label where the reactants have alkyl amino residues, again in a known manner with regard to solvents, proportions and reaction conditions. Certain bifunctional reagents, possibly glutaraldehyde may not be suitable because, while they couple, they may modify nucleic acid and thus interfere with the assay. Routine precautions can be taken to prevent such difficulties.

Also advantageously, the DNA binding ligand can be linked to the label by a spacer, which includes a chain of up to about 40 atoms, preferably about 2 to 20 atoms, including, but not limited to, carbon, oxygen, nitrogen and sulfur. Such spacer can be the polyfunctional radical of a member including, but not limited to, peptide, hydrocarbon, polyalcohol, polyether, polyamine, polyimine and carbohydrate, e.g., -glycyl-glycyl-glycyl- or other oligopeptide, carbonyl dipeptides, and omega-amino-alkane-carbonyl radical or the like. Sugar, polyethylene oxide radicals, glyceryl, pentaerythritol, and like radicals also can serve as spacers. Spacers can be directly linked to the nucleic acid-binding ligand and/or the label, or the linkages may include a divalent radical of a coupler such as dithiobis succinimidyl propionate, 1,4-butanediol diglycidyl ether, a diisocyanate, carbodiimide, glyoxal, glutaraldehyde, or the like.

Secondary probe for indirect detection of hybridization can be also detected by energy transfer such as in the "beacon probe" method described by Tyagi and Kramer, *Nature Biotech.*, 14:303-309 (1996) or U.S. Patent Nos. 5,119,801 and 5,312,728 to Lizardi et al. Any FRET detection system known in the art can be used in the present method. For example, the AlphaScreenTM system can be used. AlphaScreen technology is an "Amplified Luminescent Proximity Homogeneous Assay" method. Upon illumination with laser light at 680 nm, a photosensitizer in the donor bead converts ambient oxygen to singlet-state oxygen. The excited singlet-state oxygen molecules diffuse approximately 250 nm (one bead diameter) before rapidly decaying. If the acceptor bead is in close proximity of the donor bead, by virtue of a biological interaction, the singlet-state oxygen molecules reacts with chemiluminescent groups in the acceptor

beads, which immediately transfer energy to fluorescent acceptors in the same bead. These fluorescent acceptors shift the emission wavelength to 520-620 nm. The whole reaction has a 0.3 second half-life of decay, so measurement can take place in time-resolved mode. Other exemplary FRET donor/acceptor pairs include Fluorescein (donor) and tetramethylrhodamine (acceptor) with an effective distance of 55Å; IAEDANS (donor) and Fluorescein (acceptor) with an effective distance of 46Å; and Fluorescein (donor) and QSY-7 dye (acceptor) with an effective distance of 61Å (Molecular Probes).

Quantitative assays for nucleic acid detection also can be performed according to the present invention. The amount of secondary probe bound to a microarray spot can be measured and can be related to the amount of nucleic acid target which is in the sample. Dilutions of the sample can be used along with controls containing known amount of the target nucleic acid. The precise conditions for performing these steps will be apparent to one skilled in the art. In microarray analysis, the detectable label can be visualized or assessed by placing the probe array next to x-ray film or phosphorimagers to identify the sites where the probe has bound. Fluorescence can be detected by way of a charge-coupled device (CCD) or laser scanning.

Test samples

Any suitable samples, including samples of human, animal, or environmental (e.g., soil or water) origin, can be analyzed using the present method. Test samples can include body fluids, such as urine, blood, semen, cerebrospinal fluid, pus, amniotic fluid, tears, or semisolid or fluid discharge, e.g., sputum, saliva, lung aspirate, vaginal or urethral discharge, stool or solid tissue samples, such as a biopsy or chorionic villi specimens. Test samples also include samples collected with swabs from the skin, genitalia, or throat.

Test samples can be processed to isolate nucleic acid by a variety of means well known in the art (See generally, Ausubel (Ed.) *Current Protocols in Molecular Biology*, 2. *Preparation and Analysis of DNA* and 4. *Preparation and Analysis of RNA*, John Wiley & Sons, Inc. (2000)). It will be apparent to those skilled in the art that target nucleic acids can be RNA or DNA that may be in form of direct sample or purified nucleic acid or amplicons.

Purified nucleic acids can be extracted from the aforementioned samples and may be measured spectrophotometrically or by other instrument for the purity. For those skilled

in the art of nucleic acid amplification, amplicons are obtained as end products by various amplification methods such as PCR (Polymerase Chain Reaction, U.S. Patent Nos. 4,683,195, 4,683,202, 4,800,159 and 4,965,188), NASBA (Nucleic Acid Sequence Based Amplification, U.S. Patent No. 5,130,238), TMA (Transcription Mediated Amplification) 5 (Kwoh et al., *Proc. Natl. Acad. Sci., USA*, 86:1173-1177 (1989)), SDA (Strand Displacement Amplification, described by Walker et al., U.S. Patent No. 5,270,184), tSDA (thermophilic Strand Displacement Amplification (U.S. Patent No. 5,648,211 and Euro. Patent No. EP 0 684315), SSSR (Self-Sustained Sequence Replication) (U. S. Patent No. 6,156,508).

10 In a specific embodiment, a sample of human origin is assayed. In yet another specific embodiment, a sputum, urine, blood, tissue section, food, soil or water sample is assayed.

Kits

15 The present probes can be packaged in a kit format, preferably with an instruction for using the probes to detect a target gene. The components of the kit are packaged together in a common container, typically including written instructions for performing selected specific embodiments of the methods disclosed herein. Components for detection methods, as described herein, may optionally be included in the kit, for example, a second probe, 20 and/or reagents and means for carrying out label detection (e.g., radiolabel, enzyme substrates, antibodies, etc., and the like).

E. Exemplary embodiments

The exemplary embodiments described herein provide methods for typing a target 25 gene, for example HLA typing, using a DNA chip. Such typing can be used in construction of human bone marrow stem cell donor library and human umbilical cord blood stem cell library, organ transplantation testing, bone marrow transplantation testing, studies in autoimmune disease, virus infection, and cancer research, studies in predicting susceptibility to diseases, forensic identification, paternity determinations, and human 30 genetics studies, etc.

In one aspect, the exemplary embodiments provide a method for typing a target gene, which method comprises: a) isolating a target cell comprising a target gene from a suitable sample and obtaining a preparation comprising a target nucleotide sequence that

is at least a part of said target gene from said isolated target cell and, optionally another nucleotide sequence not related to said target gene; b) providing a chip comprising a support suitable for use in nucleic acid hybridization having immobilized thereon an oligonucleotide probe complementary to said target nucleotide sequence and at least one
5 of the following oligonucleotide control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe; and c) hybridizing said preparation obtained in step a) to said chip provided in step b) and assessing hybridization between said target nucleotide sequence and/or said another nucleotide sequence and said control probes comprised on said chip to determine the type
10 of said target gene. In some embodiments, the target gene is a HLA gene, e.g., a HLA class I or class II gene.

In one embodiment, the preparation of the target nucleotide sequence is obtained by isolating leukocyte from whole blood using magnetic microbeads, isolating nucleic acid from the leukocyte or using the leukocyte directly for a target gene amplification to
15 obtain the preparation of the target nucleotide sequence, wherein the target nucleotide sequence is a single-stranded DNA or RNA comprising a fluorescent or biotin label.

The present embodiment provides an improved method for preparation of the target nucleotide sequence. The prior methods for preparation of the target nucleotide sequence require the steps of purifying DNA from whole blood before amplification of
20 the target nucleotide sequence by PCR and purifying and denaturing the PCR product for later hybridization. See, U.S. Pat. No. 5,702,885. The present method for preparation of the target nucleotide sequence permits using leukocyte isolated from whole blood with magnetic microbeads directly as PCT template or nucleic acid isolated from the leukocyte for nucleic acid amplification to obtain single-stranded DNA or RNA
25 comprising a fluorescent or biotin label. The labeled DNA or RNA can be used for hybridization without being further purified.

In this embodiment, the single-stranded DNA or RNA can be obtained using asymmetrical PCR. The PCR amplification process can be conventional asymmetrical PCR using unequal amount of primers. The primers can be straight-chain primers or
30 have a hairpin structure. The primers used in the asymmetrical PCR can have same or different T_m values. The difference between the T_m value of two or more primers used in the asymmetrical PCR can range from about 1°C to about 20°C. One of the primers can have a lower T_m value to allow amplification of double-stranded product. The other

primer can have a higher T_m value to allow amplification of single-stranded DNA after obtaining certain amount of double stranded-DNA. The single-stranded target nucleotide sequence generated by this PCR amplification process can be used directly for hybridization without further purification. Example 1 demonstrated that the
5 single-stranded HLA target nucleotide sequence obtained by this method could be used for DNA chip hybridization without further purification.

Alternatively, single-stranded RNA can be obtained by transcription-mediated amplification (TMA) method. A primer comprising a T7 promoter can be included for amplification and a single-stranded RNA can be synthesized by T7 RNA polymerase.
10 Using single-stranded DNA or RNA for hybridization avoids the step of purifying and denaturing the PCR amplified product and problem of weak or loss of signal while using double-stranded DNA for hybridization.

In this embodiment, the magnetic microbeads is prepared by the methods disclosed in CN Pat. NO. 01134861.5.

15 The method disclosed herein for the preparation of a target nucleotide sequence can be used for fast nucleic acid sample preparation and for active biochip operation in order to construct a micro-total analysis system (or lab-on-a-chip).

The PCR system and process is further detailed in Example 1. The PCR amplification product is shown in Figure 1 and Figure 2.

20 In another embodiment, the chip of the invention is constructed by designing oligonucleotide probes according to the published HLA allele gene sequences, and immobilizing the probes on chemically modified surface of the chip.

In this embodiment, the probes are designed according to published HLA allele gene sequence for middle-level resolution HLA typing and immobilized on chemically
25 modified support of the chip. The probes can be positive-stranded or negative-stranded probes. The probes can have about 10-30 nucleotides. The HLA allele gene sequence can be first analyzed by a sequence analysis software to identify single nucleotide polymorphism (SNP) site, and probe sequence covering the SNP site is designed based on the required T_m value. Examples of the probes used for the invention are shown in
30 Table 1.

In this embodiment, the probes can be immobilized on the support of the chip at a temperature higher than room temperature to increase immobilization efficiency. Higher immobilization efficiency saves the amount of probes to be used and reduces the

cost of chip construction. The prior methods for immobilizing probes use room temperature or close to room temperature. The present embodiment uses higher temperature to immobilize probes having NH₂ modification at one end of the probe because the reaction between NH₂ group and CHO group to form -NH-CO- bond is more efficient at a higher temperature.

In another embodiment, a method is provided for assessing the quality of the probes, such as purity and the terminal modification, via DHPLC. Terminal modification efficiency is an important indication for the quality of the probes. DHPLC can separate terminally modified and unmodified probes and thus can be used for quality control of the probes. An example of using DHPLC for assessing terminal NH₂ modification of probes is shown in Table 2. DHPLC can also be used to assess nucleotide mutations or deletions in the probes.

In another embodiment, a method comprising hybridizing the preparation comprising the target nucleotide sequence to the chip, washing the chip with a solution, scanning the chip after hybridization for signals, and assessing hybridization between the target nucleotide sequence and the probes immobilized on the chip to determine the type of the target gene.

In this embodiment, hybridization condition and washing condition that give strong signal and minimize possibility of false positive signal are selected. The probes used for the invention are selected from sequences according to human HLA allele gene sequence provided from the Twelfth International Conference of Histocompatibility (ftp.ebi.ac.uk) and cloned standard HLA allele gene. The cloned standard HLA allele genes were sequenced and many sequences did not match sequences provided by the Twelfth International Conference of Histocompatibility. A HLA allele gene library was constructed for designing probes of the invention.

In another embodiment, a series of control probes and a pattern of probe arrangement shown in Table 3 are provided to assess the quality of the chip and reliability of the result from the hybridization process. The arrangement of probes shown in Table 3 can be used for typing a target gene, including but not limited to HLA typing. Multiple copies (for example, 1-10 copies) of a probe can be immobilized on the chip. The multiple copies of the probe can be immobilized adjacently or separately on the chip. One to ten copies of a positive control probe can be immobilized on the chip and the variations in the length and sequence of the immobilized positive control

probes, when hybridized with the target nucleotide sequence, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. The negative control probe has about 1-3 basepair mismatches when compared to the positive control probe. The positive or the negative control probe can be complementary to a portion of the target nucleotide sequence, a nucleotide sequence amplified synchronically with the target nucleotide sequence, or a synthetic nucleotide sequence. The hybridization control probe can be complementary to a synthetic nucleotide sequence not related to the target gene. The hybridization control probe can be complementary to a synthetic labeled nucleotide sequence or has about 1-2 basepair mismatches when compared to the synthetic labeled nucleotide sequence. The immobilization control probe is a probe that is chemically modified at one end (for example, NH_2 modified) and has a detectable label (for example, fluorescent label) at the other end. The immobilization control probe is complementary to a synthetic nucleotide sequence not related to the target gene. The positive control probe, the negative control probe, the hybridization control probe, and the immobilization control probe can be immobilized on the four corners of the chip, in the center of the chip, or have any suitable orderly or random immobilization pattern. This pattern arrangement for the control probes can be used, but not limited to, for HLA typing.

In another embodiment, a method for data analysis is provided for assessing the hybridization signal which can be used for typing any target gene, including but not limited to typing a HLA gene. The method includes assessment of the immobilization efficiency, the overall hybridization efficiency, the hybridization specificity, and the positive signal and the negative signal. The overall hybridization efficiency can be assessed by analyzing a signal from the immobilization control probe, analyzing the hybridization between the hybridization control probe and a labeled synthetic nucleotide sequence not related to the target gene, and analyzing the hybridization signal involving the positive control probe and the negative control probe. The hybridization specificity can be assessed by analyzing the ratio between the hybridization signal involving the positive control probe and the hybridization signal involving the negative control probe, and the ratio between the hybridization signal involving the positive hybridization control probe and the hybridization signal involving the negative hybridization control probe, and increased ratios indicating the increased hybridization specificity. In hybridizations involving a group of closely related probes, a positive signal(s) can be determined based on

the following criteria: a) the ratio of the hybridization signal over background noise is more than 3; b) the ratio of the hybridization signal over a relevant positive control probe hybridization signal is within a predetermined range; c) comparing hybridization signals of all probes giving positive signals based on the steps of a) and b), or hybridization signals of two probes giving two strongest hybridization signals when only one probe giving positive signal based on the steps of a) and b), to determine whether the signal is positive or negative; and d) there are 2 or less than 2 positive signals involving the group of closely related probes.

After the hybridization, the chip can be scanned and the data can be analyzed. A target gene type, for example, a HLA gene, can be determined using a data analyzing software.

F. Example

Example 1

To isolate a target cell for typing a target HLA gene, 5 ul of whole blood with anti-agglutination agent in ACD (23 mM citric acid, 80 mM glucose, and 45 mM sodium citrate) were mixed gently with 20 ul magnetic beads (which is in TE buffer pH6.0 at 15 mg/ml) in a 1.5 ml Eppendorf tube on rotating shaker for 10 seconds and allowed to settle for 3 minutes. The magnetic beads were then immobilized by a magnetic stand and the supernatant was discarded. The magnetic beads were washed two times with PBS. After wash, the magnetic beads were resuspended in 100 ul TE buffer, 5 ul of the resuspended magnetic beads were used as template for PCT amplification. PCR was carried out in 25 ul of PCT reaction solution made by mixing 2.5 ul of 10x buffer, 0.5 ul of 10 mM dNTP, 0.5 ul of 1 mM Cy5 labeled dCTP, 0.5 ul of 100 ng/ul template DNA, 0.5 ul of 1 uM upstream primer, 2.5 ul of 10 uM downstream primer, 0.5 ul of 5 U/ul Taq polymerase, and sterile double distilled water to make final reaction volume to 25 ul. The PCT amplification process included 3 minutes at 96°C; twenty six cycles of 25 second at 96°C, 45 second at 71°C, and 30 second at 72°C; nine cycles of 25 second at 96°C, 60 second at 68°C, and 120 second at 72°C; followed by 8 minutes at 72°C.

After the PCT amplification, the reaction product may be analyzed by 1.2% agarose gel electrophoresis. The reaction product may be used directly for the following hybridization reaction without being analyzed.

To perform hybridization, 5 ul of PCT reaction product, after being mixed with 3 ul

hybridization solution, were incubated at 98°C for 5 minutes. The hybridization reaction was then carried out at 65°C for 1 hour to allow hybridization of PCT reaction product with probes immobilized on a DNA chip. After hybridization, unreacted PCT reaction product on the chip was washed off using deionized water. The chip was
 5 further washed for 10 minutes at 45°C in washing buffer and rinsed in deionized water. The chip was then dried.

The hybridized chip was scanned by a specialized scanner and a hybridization pattern was obtained (Fig. 3). The hybridization pattern was analyzed using a specialized software to generate a database. The database was analyzed and genetic
 10 typing of the target gene was obtained.

Example 2

To determine quality of probes, two oligonucleotide probes (PBH_0303019 and PBH_0301119) that had NH₂ modification at one end at 10 uM in H₂O, were applied to
 15 WAVE® Nucleic Acid Fragment Analysis System (Transgenomic, USA). The column temperature was set at 80°C and the probes were washed in gradient acetonitrile buffer. The eluant was detected at 260 nM using an ultra violet detector. The number and shape of the peaks eluted over time indicated the quality of the probes. As shown in Figure 5, the useful content of 5A and 5B is 93.35% and 64.8% respectively.

20

Example 3

The probes in table 1 were used as HLA_A, B, DRB1 genotyping probes. PBH_0301xxx represent the HLA-A probes, PBH_0302xxx represent HLA-B probes and PBH_0303xxx represent the HLA-DRB1 probes.

Example 4

25 Cloning of HLA standard alleles, the procedure are as following:

```

      PCR of objected fragment from genomic DNA
      ↓
electrophoresis of PCR products using agarose gel
      ↓
30  purify the PCR products using gel recovering
      ↓
      linking reaction
  
```

↓
translation reaction
↓
identify the colonies by PCR (M13 primer)
↓
5 culture the positive colonies on LB substrate, 37°C over night
↓
identify the culture solution using PCR (HLA universal primer and
sequence specific primer)
↓
10 sequencing of the positive colonies and keep the strain

A HLA standard gene bank was constructed for detecting the HLA_A, B, DRB1 genotyping probes and QC of HLA chips. There are 43 HLA-A alleles have been cloned which distributed to 19 allele groups and covering 95.0% of the medium types; For HLA_B, 47 alleles have been cloned which distributed to 29 allele groups and covering 80% of the medium types; For HLA_DR, 22 alleles have been cloned which contributed to 14 allele groups and covering 87.5% of the medium types. The probes in table 1 were designed based on the allele sequences of 12th IHWG(www.ihwg.org). Then the probes were immobilized on chemical modified slides to fabricate HLA chips.

The hybridization and washing conditions should be keep a strong positive signal and a relatively weak false positive signal. The temperature of hybridization and washing were 65°C and 45°C respectively, and the ion strength was 0.2%SDS, 0.1xSSC.

The samples containing target gene were hybridized with HLA chips, then through washing and scanning the data were gain and the genotype was detected. For example, 5µl PCR product and 5µl hybridization buffer were mixed then hybridized 1 hr at 65°C, and then washing the chip using a solution containing 0.2% SDS and 0.1xSSC 10 min at 45°C, washing 5 min again using 1xSSC, rinsed in water for 1 min. Then the chip was dried with centrifuging, scanned with a scanner. The photo was treated and the data were analyzed using HLA specific software and the genotype result was produced.

The probes for HLA-A, B, DRB1 genotyping have been shown in table1.

Table 1. The genotyping probes for HLA_A, B, DRB1 locus

HLA_A probes

Probe name	sequences
PBH_0301001	CCTGCGCTCTTGGACCGC
PBH_0301001a	CCTGCGCTCTTGGACCGC
PBH_0301001b	CCTCCTGCGCTCTTGGACCG
PBH_0301001c	CCTGCGCTCTTGGACC
PBH_0301001d	CGTGTCCCGGCCCGGC
PBH_0301001e	ATGGAGCCGCGGGCGC
PBH_0301001g	CCTGCGCTCTTGGACCGCGG
PBH_0301001comp	GCGGTCCAAGAGCGCAGG
PBH_0301002a	CCTGCGCTTTTGGACCGC
PBH_0301002B	CCTGCGCTGTTGGACCGC
PBH_0301003	GCAGGAGAGGCCTGAGTATTGG
PBH_0301004	CACCATCAGATAATGTATGGCTGC
PBH_0301004	CACCATCCAGATAATGTATGGCTGC
PBH_0301101	TTCTACACCTCCGTGTCCCG
PBH_0301103	CGCTTCATCGCAGTGGGCT
PBH_0301105	CGAGCCAGAAGATGGAGCC
PBH_0301106	CCGCGGGCACCGTGGATA
PBH_0301107	GCAGGAGGGTCCGGAGTATT
PBH_0301111	GACGTGGGGCCGGACGGG
PBH_0301112	GACGGGGCCTCCTCCGC
PBH_0301114	CGGGTACCACAGTACGCCT
PBH_0301115	GGTACCGGCAGGACGCCTA
PBH_0301116	CGCCCTGAACGAGGACCTG
PBH_0301117	CGGACATGGCAGCTCAGATC
PBH_0301119	CCACCAAGCACAAGTGGGA
PBH_0301120	AAGTGGGAGACGGCCCATG
PBH_0301121	AGGCGGCCCGTGTGGCGG
PBH_0301122	AGGCGGTCCATGCGGCGG
PBH_0301123	CGGCCCATGAGGCGGAGC
PBH_0301125	TACCTGGATGGCACGTGCG
PBH_0301127	CTGGAGGGCGAGTGCGTGG

PBH_0301128	TGCGTGGACGGGCTCCGC
PBH_0301129	GTATTTCTACACCTCCGTGTCCCG
PBH_0301130	CGAGCGGTTTGACAGCGAC
PBH_0301131	CGTGCGGTTGACAGCGAC
PBH_0301133	CGTGGGGCCGACGGG
PBH_0301136	AGGCGGTCCATGCGGCG
PBH_0301137	CCCGGCCGCGGGAGCCC
PBH_0301138	CCGCGGGCGCGTGGATA
PBH_0301139	TGGGACGAGGACAGGGA
PBH_0301140	TGGGACCAGGACACGGA
PBH_0301141	TGGGACCCTGCGCGGCTA
PBH_0301142	GACGTGGGGTCGGACGGG
PBH_0301143	GACGGGCGCTTCTCCGC
PBH_0301144	GCGGGTACCAGCAGGACGC
PBH_0301145	CGCCCTGAAAGAGGACCTG
PBH_0301146	AGCTCAGATCACCAAGCGCA
PBH_0301146a	TCAGATCACCAAGCGCAAGAG
PBH_0301147	AGCTCAGATCACCGAGCGCA
PBH_0301148	GGCTCAGATCACCCAGCGCA
PBH_0301148a	TCAGATCACCCAGCGCAAGTG
PBH_0301149	AGACGGCCCATGAGGCG
PBH_0301149a	AGACGGCCCATGAGGCGG
PBH_0301150	GCGGAGCAGCGGAGAGTCT
PBH_0301150a	AGACGGCCCATGAGGCGG
PBH_0301151	GCGGAGCAGTTGAGAGCCT
PBH_0301151a	GGCGGAGCAGTTGAGAGCC
PBH_0301152	GCGGAGCAGTGAGAGCCT
PBH_0301153	TACCTGGAGGGCACGTGCG
PBH_0301154	TGCGTGGAGTGGCTCCGC
PBH_0301155	TCACCGAGTGGACCTGGGG
PBH_0301155a	CCGAGTGGACCTGGGGACC
PBH_0301156	TGACCGAGAGAACCTGCGG

PBH_0301156a	CCGAGAGAACCTGCGGATCG
PBH_0301157	GAAGGCCCACTCACAGACTG
PBH_0301171	TATTTCTTCACATCCGTGTCCCG
PBH_0301172	TCTACACTTCCGTTTCCCGGC
PBH_0301173	CTACACCTCCATGTCCCGGC
PBH_0301174	CCGGAACACACGAAAGTGAA
PBH_0301175	ATTGGGACGGGAGACACG
PBH_0301176	GACACGGAATATGAAGGCCA
PBH_0301177	GACACGGAATGTGAAGGCC
PBH_0301178	TCACAGACTCACCGAGTGGACC
PBH_0301179	TCACAG ATTGACCGAGTGGACC
PBH_0301180	TCACAG ACTGACCGAGTGGACC
PBH_0301181	CGAGCGAACCTGGGGACC
PBH_0301182	CCGAGAGAGCCTGCGGATC
PBH_0301183	ACCGAGAGAACCTGGGGACC
PBH_0301184	GTGGACCTGGCGACCCTGC
PBH_0301185	CACCGTCCAGAGGATGTATGGC
PBH_0301186	ACCAGCAGGACGCTTACGACG
PBH_0301187	TCGCCTTGAACGAGGACCTG
PBH_0301188	CCTGCGCTCTTGACCGC
PBH_0301189	TCAGACCACCAAGCACAAGTGG
PBH_0301190	GAGGCGGCCCATGTGGC
PBH_0301191	GGCCCATGCGGCGGAGC
PBH_0301192	GCGGCCCGTCGGGCGGA
PBH_0301193	GCACGTGCGTGGAGTGGC
PBH_0301194	GCCGGTGCGTGGACGGGC
PBH_0301195	GGCGAGTGCGTGGAGTGGC
PBH_0301196	GCACGTGCGTGGACGGGC
PBH_0301197	GCCGGTGCGTGGAGTGGC
PBH_0301198	GGCGAGTGCGTGGACGGGC
PBH_0301199	AGACACGGAAGTGAAGGCC
HLA_Bprobe	

Probe name	sequences
PBH_0302001(positive)	TGGCCCTGACCGAGACCTGGGC
PBH_0302001a	CTACAACCAGAGCGAGGCCG
PBH_0302002(negative)	GCCCTGACCCAGACCTGGG
PBH_0302003	CCCGAACCTCCTCCTGC
PBH_0302004	CCCGAACCGTCCTCCTGC
PBH_0302005	TGCTCTCGGCGGCCCTG
PBH_0302006	TGCTCTCGGGAGCCCTGG
PBH_0302007	GGGGGGCAGTGGCCCT
PBH_0302008	TGAGGTATTTGACACCGCCA
PBH_0302009	TGAGGTATTTCTACACCGCCATG
PBH_0302010	TTCCACACCTCCGTGTCCC
PBH_0302011	TCTACACGCCATGTCCCG
PBH_0302012	TCTACACCTCCGTGTCCCGG
PBH_0302013	CCGCTTCATCTCAGTGGGCTAC
PBH_0302014	CGCTTCATCACCGTGGGCT
PBH_0302015	CGCTTCATCGCAGTGGGCT
PBH_0302016	TACGTGGACGGCACCCAGTT
PBH_0302017	CGTGGACGACCCAGTTCG
PBH_0302018	GGACGACAGCTGTTCGTGA
PBH_0302019	TGGACGACAGCAGTTCGTG
PBH_0302020	GCGACGCCACGAGTCCG
PBH_0302021	GCGACGCCGCGAGTCC
PBH_0302022	GAGTCCGAGAGAGGAGCCGC
PBH_0302023	CCGAGGAAGGAGCCCGG
PBH_0302024	AGGATGGCGCCCCGG
PBH_0302025	GGACGGAGCCCCGGGC
PBH_0302026	CGGGCGCCGTGGATAGAG
PBH_0302027	CGGGCGCCATGGATAGAG
PBH_0302028	GGGGCCGAATATTGGGAC
PBH_0302029	GGGGCCGAGTATTGGGAC

PBH_0302030	GGGACCGGGAGACACAGATCT
PBH_0302031	TGGGACCGGAACACACAGATC
PBH_0302032	ACACAGAAGTACAAGCGCCAGG
PBH_0302033	ACACGGAACATGAAGGCCTCC
PBH_0302034	CACACAGATCTTCAAGACCAACAC
PBH_0302035	ATCTGCAAGGCCAAGGCACA
PBH_0302036	TACAAGGCCCAGGCACAGACT
PBH_0302037	ACACAGACTGACCGAGAG
PBH_0302038	CACACAGACTTACCGAGAGAGCC
PBH_0302039	GCACCGCGCTCCGCTA
PBH_0302040	CGGACCCTGCTCCGCTACT
PBH_0302041	ACCTGCGGATCGCGCTC
PBH_0302042	CGGAACCTGCGCGGCT
PBH_0302043	CGGGTCTCACATCATCCAGAGG
PBH_0302044	GGGTCTCACACCCTCCAGAGG
PBH_0302045	TCCACTTGGCAGACGATGTATG
PBH_0302046	ACACCCTCCAGAGGATGTACGG
PBH_0302047	CGACCTGGGGCCCGAC
PBH_0302048	CGACGTGGGGCCGGAC
PBH_0302049	GGGTACCACCAGGACGCCT
PBH_0302050	CGGGTATGACCAGGACGCC
PBH_0302051	GGGCATGACCAGTCCGCC
PBH_0302052	GCGGGTATAACCAGTTCGCC
PBH_0302053	GAGGACCTGCGCTCCTGGA
PBH_0302054	GAGGACCTGAGCTCCTGGA
PBH_0302055	GGACCGCCGCGGACAC
PBH_0302056	GGACCGCGGCGGACAC
PBH_0302057	CGGACACGGCGGCTCAG
PBH_0302058	CGGACACCGCGGCTCAG
PBH_0302059	GGCCCGTGAGGCGGAG
PBH_0302060	GGCCCGTGTGGCGGAG
PBH_0302061	GCGGAGCAGGACAGAGCCTA

PBH_0302062	GCGGAGCAGTGGAGAGCCTA
PBH_0302063	GCGGAGCAGCTGAGAGCCTA
PBH_0302064	AGCAGCTGAGAACCTACCTGGAG
PBH_0302065	AGCAGCTGAGAGCCTACCTGGAG
PBH_0302066	GGAGGGCGAGTGCCTGG
PBH_0302067	GGAGGGCACGTGCCTGG
PBH_0302068	GGAGGGCCTGTGCCTGG
PBH_0302069	CGTGGAGTCGCTCCGCAG
PBH_0302070	CGTGGAGTGGCTCCGCAG
PBH_0302071	CTCCGCAGACCTGGAGAAC
PBH_0302072	GCTCCGCAGATACCTGGAGAA
PBH_0302073	AGGACAAGCTGGAGCGCG
PBH_0302074	GGACACGCTGGAGCGCG
PBH_0302075	GGAGACGCTGCAGCGCG
HLA_DRB1probe	
Probe name	sequences
PBH_0303001	CTTGTGGCAGCTTAAGTTGAATGT
PBH_0303002	TGGAGTACTCTACGTCTGAGTGCA
PBH_0303003	GGAGCAGGTAAACATGAGTGT
PBH_0303004	CCTGTGGCAGGGTAAGTATAAGT
PBH_0303005	TTGGAGTACTCTACGGGTGAGTG
PBH_0303006	CCTGTGGCAGCCTAAGAGGG
PBH_0303007	CCTGGAGCAGGCGCGG
PBH_0303008	CCTGGAAGACGAGCGGGC
PBH_0303009	CCAGGAGGAGAACGTGCGC
PBH_0303010	CCTGGAAGACAGGCGGGC
PBH_0303011	CGGTTGCTGGAAAGATGCATC
PBH_0303012	CGGTTCTGGACAGATACTTCTATCAC
PBH_0303013	TGCAGTTCCTGGAAAGACTCTTCT
PBH_0303014	CGGTATCTGCACAGAGGCATCT
PBH_0303015	TGCTGGAAAGACGCGTCCA
PBH_0303016	CGGTTACTGGAGAGACACTTCCATA

PBH_0303017	CGGCCTGATGAGGAGTACTGG
PBH_0303018	CCTGTGCGCCGAGTCCTGGA
PBH_0303019	GGCCTGATGCCGAGTACTGG
PBH_0303020	CAGGAGGAGCTCCTGCGCTT
PBH_0303021	GAGCAGAAGCGGGGCCGG
PBH_0303022	TCCTGGAGCGGAGGCGG
PBH_0303023	GCGGGCCCTGGTGACA
PBH_0303024	GGGGGAGTTCCGGGCGG
PBH_0303025	GGGGGAGTACCGGGCGG
PBH_0303026	GGCCTGACGCTGAGTACTGG
PBH_0303027	CAATGGGACGGAGCGGGTGC
PBH_0303027a	AATGGGACGGAGCGGGTG
PBH_0303027b	GGGACGGAGCGGGT
PBH_0303028	GGGGGAGTTCCGGGCG
PBH_0303029	TGGGGGAGTACCGGGCG
PBH_0303030	ACCAAGAGGAGTACGTGCGCTT
PBH_0303031	GCCTGCTGCGGAGCACTG
PBH_0303032	CCAGGAGGAGTTCGTGCGC
PBH_0303033	CCTGGAAGACGAGCGGGC
PBH_0303034	GCCTGCTGCGGAGCACTG
PBH_0303035	GGCCTGATGCCGAGTACTGG
PBH_0303036	CCAGGAGGAGAACGTGCGC
PBH_0303037	CCTGGAAGACGAGCGGGC
PBH_0303038	GACAGGCGCGCCGCG
PBH_0303039	CTGGAGCAGAGCGGGC
PBH_0303040	AACCAAGAGGAGTACGTGCGC
PBH_0303041	AATGGGACGCAGCGGBT
PBH_0303055	CATCCTGGAAGACGAGCGGGG

Example 5

An array of HLA typing, there are 64 detection probes, 2 positive probes, 1 negative probes, 1 hybridization control negative probes, 1 hybridization

control positive probes. As shown in table 2, the italic letters represent the control probes and the others are detection probes. Table 3 has shown an example of the arrangement of the probes in HLA chip.

5 Table 2. The arrangement of the probes in a microarray

<i>Hybridization Control (positive)</i>	<i>Hybridization Control (positive)</i>	Detection probe 1	Detection probe 1	Detection probe 2	Detection probe 2	Detection probe 3	Detection probe 3	Detection probe 4	Detection probe 4	<i>Hybridization Control (positive)</i>	<i>Hybridization Control (positive)</i>
<i>Hybridization Control (negative)</i>	<i>Hybridization Control (negative)</i>	Detection probe 5	Detection probe 5	Detection probe 6	Detection probe 6	Detection probe 7	Detection probe 7	Detection probe 8	Detection probe 8	<i>Hybridization Control (negative)</i>	<i>Hybridization Control (negative)</i>
<i>Positive (1)</i>	<i>Positive (1)</i>	Detection probe 9	Detection probe 9	Detection probe 10	Detection probe 10	Detection probe 11	Detection probe 11	Detection probe 12	Detection probe 12	<i>positive (1)</i>	<i>positive (1)</i>
<i>Positive (2)</i>	<i>Positive (2)</i>	Detection probe 13	Detection probe 13	Detection probe 14	Detection probe 14	Detection probe 15	Detection probe 15	Detection probe 16	Detection probe 16	<i>positive (2)</i>	<i>positive (2)</i>
<i>negative</i>	<i>negative</i>	Detection probe 17	Detection probe 17	Detection probe 18	Detection probe 18	Detection probe 19	Detection probe 19	Detection probe 20	Detection probe 20	<i>negative</i>	<i>negative</i>
Detection probe 53	Detection probe 53	Detection probe 21	Detection probe 21	Detection probe 22	Detection probe 22	Detection probe 23	Detection probe 23	Detection probe 24	Detection probe 24	Detection probe 57	Detection probe 57
Detection probe 54	Detection probe 54	Detection probe 25	Detection probe 25	Detection probe 26	Detection probe 26	Detection probe 27	Detection probe 27	Detection probe 28	Detection probe 28	Detection probe 58	Detection probe 58
Detection	Detection	Detection	Detection	Detection	Detection	Detection	Detection	Detection	Detection	Detection	Detection

probe 55	probe 55	probe 29	probe 29	probe 30	probe 30	probe 31	probe 31	probe 32	probe 32	probe 59	probe 59
Dete ction probe 56	Detec tion probe 56	Detec tion probe 33	Detec tion probe 33	Detec tion probe 34	Detec tion probe 34	Detec tion probe 35	Detec tion probe 35	Detec tion probe 36	Detec tion probe 36	Detec tion probe 60	Detec tion probe 60
<i>negat ive</i>	<i>negati ve</i>	Detec tion probe 37	Detec tion probe 37	Detec tion probe 38	Detec tion probe 38	Detec tion probe 39	Detec tion probe 39	Detec tion probe 40	Detec tion probe 40	<i>negat ive</i>	<i>negat ive</i>
<i>Posit ive (2)</i>	<i>Positi ve (2)</i>	Detec tion probe 41	Detec tion probe 41	Detec tion probe 42	Detec tion probe 42	Detec tion probe 43	Detec tion probe 43	Detec tion probe 44	Detec tion probe 44	<i>Positi ve (2)</i>	<i>Positi ve (2)</i>
<i>Posit ive (1)</i>	<i>Positi ve (1)</i>	Detec tion probe 45	Detec tion probe 45	Detec tion probe 46	Detec tion probe 46	Detec tion probe 47	Detec tion probe 47	Detec tion probe 48	Detec tion probe 48	<i>Positi ve (1)</i>	<i>Positi ve (1)</i>
<i>Hybr idizat ion Contr ol (nega tive)</i>	<i>Hybri dizati on Contr ol (nega tive)</i>	Detec tion probe 49	Detec tion probe 49	Detec tion probe 50	Detec tion probe 50	Detec tion probe 51	Detec tion probe 51	Detec tion probe 52	Detec tion probe 52	<i>Hybri dizati on Contr ol (nega tive)</i>	<i>Hybri dizati on Contr ol (nega tive)</i>
<i>Hybr idizat ion Contr ol (posit ive)</i>	<i>Hybri dizati on Contr ol (posit ive)</i>	Detec tion probe 61	Detec tion probe 61	Detec tion probe 62	Detec tion probe 62	Detec tion probe 63	Detec tion probe 63	Detec tion probe 64	Detec tion probe 64	<i>Hybri dizati on Contr ol (posit ive)</i>	<i>Hybri dizati on Contr ol (posit ive)</i>

Table 3. The arrangement of the probes in a HLA typing array

Hex	Control		A062-1			IC1		
A001comp A001comp A001comp	blank blank blank		175 175 175			A03Control1 A03Control1 A03Control1		
A030	A009		139 139 139			IC2		
159a 159a 159a	171a 171a 171a		A062-2			A03Control3 A03Control3 A03Control3		
159b 159b 159b	129 129 129		139b 139b 139b			A001a A001a A001a		
A001 A001 A001	172 172 172		139a 139a 139a			A001 A001 A001		
A076	A114		140 140 140			Negative Negative Negative		
156 156 156	115 115 115		A063			A002b A002b A002b		
181 181 181	144 144 144		174a 174a 174a			A070		
182 182 182	114 114 114		174b 174b 174b			157 157 157		
A074	A080		A148			162 162 162		
178 178 178	141a 141a 141a		190 190 190			A156		
180a 180a 180a	141 141 141		192 192 192			151b 151b 151b		
Negative	A111		121a 121a 121a			152a 152a 152a		
A002b A002b A002b	143a 143a 143a		A161			152b\k29698 152b\k29698 152b\k29698		
A001 A001 A001	112 112 112		125 125 125					
A001b A001b A001b	A142		153 153 153			A001 A001 A001		
IC2	148b 148b 148b		A163			A166		
A03Control3 A03Control3 A03Control3	146b 146b 146b		195b 195b 195b			128 128 128		
IC1	Control		195a 195a 195a			154 154 154		
A03Control1 A03Control1 A03Control1	blank blank blank					Hex Hex		
						A001comp A001comp A001comp		

The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.